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September 2, 2009

VIA OVERNIGHT MAIL
TSCA Department Processing Center, Rm. 6248
Office of Pollution Prevention and Toxics
U.S. Environmental Protection Agency
1201 Constitution Ave., N.W.
Washington, D.C. 20004

Company Sanitized

J09-4

314-418-4900

To Whom It May Concern:

Novozymes North America, Inc. submits the enclosed Microbial Commercial Activity Notice (MCAN) for a new microorganism, *Trichoderma reesei*. This MCAN has been assigned the identifying number **TS F292TR**.

There are four copies of this MCAN, three unsanitized copies containing marked confidential business information (CBI) (highlighted in yellow) and a sanitized copy from which all confidential business information has been removed. A limited amount of information submitted in this MCAN has been claimed as confidential business information and substantiation for these claims is given in the document. Also, the company identified in this notice has remitted the fee of $2,500.00 to the St. Louis, MO mailing addressed as specified in 40CFR §700.45 and instructed on the EPA's PMN Fees website.

We believe this submission to be complete in that all information of potential importance to human health and environmental safety has been provided. The information supplied should be sufficient to allow the Agency to make an informed evaluation of the safety of this new microorganism and to add it to the list of TSCA Inventory of Chemical Substances.

Please contact me by direct telephone at 919 494-3152; direct fax at 919 494-3420; or email at DCBe@novozymes.com if you have questions or require further information.

Respectfully submitted,

Denise Bernstein

Laws, regulations and third party rights may prevent customers from importing, processing, applying and/or reselling certain products in a given manner. It is the responsibility of the customers that their specific use of products from Novozymes does not infringe relevant laws and regulations and, furthermore, does not infringe patents or other third party rights. Unless separate agreements exist, the contents of this document are subject to change without further notice.

Novozymes North America, Inc.
Regulatory Affairs
77 Perry Chapel Church Road, P.O. Box 576
Franklinton, North Carolina 27525

[redacted]

Tel:919-494-3000 ***Fax: 919-494-3420*** ***www.novozymes.com***

Confidential Business Information
2009-21789

Microbial Commercial Activity Notice (MCAN):

Contained Use of a Genetically Modified Microorganism

Trichoderma reesei

for the Biosynthesis of a Cellulose-Degrading Protein Preparation

TS F292TR

Novozymes North America, Inc.
Denise Bernstein
Date: 27 August 2009

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CERTIFICATION STATEMENT

I certify that to the best of my knowledge and belief:

1. The company named in this submission intends to manufacture, import, or process for a commercial purpose, other than in small quantities solely for research and development, the microorganism identified in this submission.

2. All information provided in this submission is complete and truthful as of the date of submission.

3. I am including with this submission all test data in my possession or control and a description of all other data known to or reasonably ascertained by me as required by 40 CFR 725.160.

4. The company identified in this notice has remitted the fee ($2,500.00) specified in 40 CFR 700.45(b).

Signature of authorized official:

Date: 9/2/2009

Denise Bernstein, M.S., M.B.A.
Staff Specialist
Novozymes

CONFIDENTIAL BUSINESS CLAIMS

A limited amount of information submitted in this MCAN is claimed as Confidential Business Information (CBI). The CBI has been limited to the following two categories of information:

Codes used in text margins to identify CBI

1. Microorganism Identity and Construction **M.I.**
2. Production Volume **P.V.**
3. Production Information **P.I.**

Highlighted **M.I., P.V., and P.I.** sections are identified by yellow.

SUBSTANTIATION: **1. Microorganism Identity and Construction M.I.**

Substantiation of the combined CBI information, that is marked '**M.I.**' in the text, is given in the following, addressing both the general and the specific questions as required.

(c)(1). For what period of time is a claim of confidentiality being asserted? If the claim is to extend until a certain event or point in time, indicate that event or time period. Explain why the information should remain confidential until such point.

The information is claimed CBI for an unlimited period of time. The information, marked '**M.I.**' in the text, is claimed as Confidential Business Information because it concerns details regarding the rDNA construction of the production strain and, therefore, represents a core technology base of Novozymes. While individual steps in the rDNA construction might be well known or publicly available information, the combination of steps constitutes the information that is claimed as CBI.

(c)(2). Briefly describe any physical or procedural restrictions within the company or institution relating to the use and storage of the information claimed as confidential. What other steps, if any, apply to use or further disclosure of the information?

The information is designated confidential and is only distributed internally to persons who have a documented need in order for them to perform appropriate risk assessment or to obtain necessary approvals by authorities.

(c)(3). Has the information claimed as confidential been disclosed to individuals outside of the company or institution? Will it be disclosed to such persons in the future? If so, what restrictions, if any, apply to use or further disclosure of the information?

The information will only be disclosed to individuals outside the company if this is a necessary part of obtaining approvals by authorities or business agreements. If the information is to be disclosed to such persons, it will only be done under a confidentiality claim or a secrecy agreement.

(c)(4). Does the information claimed as confidential appear, or is it referred to, in any of the following questions? If the answer is yes to any of these questions, indicate where the information appears and explain why it should nonetheless be treated as confidential. (i) Advertising or promotional materials for the microorganism or the resulting end product?
(ii) Material safety data sheets or other similar materials for the microorganism or the resulting end product?
(iii) Professional or trade publications?

(iv) Any other media available to the public or to competitors?
(v) Patents?
(vi) Local, State, or Federal agency public files?

The production organism has been patented/patent pending.

(c)(5). Has EPA, another Federal agency, a Federal court, or a State made any confidentiality determination regarding the information claimed as confidential? If so, provide copies of such determinations.

No.

(c) (6). For each type of information claimed confidential, describe the harm to the company's or institution's competitive position that would result if this information were disclosed. Why would this harm be substantial? How could a competitor use such information? What is the causal connection between the disclosure and harm?

The information represents the state-of-the-art of one of Novozymes' core technologies that has been obtained as a result of substantial investments in research and development within rDNA technology.

(c) (7). If EPA disclosed to the public the information claimed as confidential, how difficult would it be for the competitor to enter the market for the resulting product? Consider such constraints as capital and marketing cost, specialized technical expertise, or unusual processes.

A competitor, already using rDNA technology, would be significantly eased in constructing a similar production strain. It is anticipated that commercialisation would be fairly easy as the proteins produced by the production strain are similar to the protein products currently manufactured and marketed by our competitors.

(d) (1). Has the microorganism or method of production been patented in the U.S. or elsewhere? If so, why is confidentiality necessary?

The microorganism that is the subject of this notice has not been patented. A component used in the construction of the microorganism, however, has a patent pending (see (c) (4) above)).

(d) (2). Does the microorganism leave the site of production or testing in a form which is accessible to the public or to competitors? What is the cost to a competitor, in time and money, to develop appropriate use conditions? What factors facilitate or impede product analysis?

The microorganism is removed from the final protein product.

(d) (3). For each additional type of information claimed as confidential, explain what harm would result from disclosure of each type of information if the identity of the microorganism were to remain confidential.

Not applicable.

(e). Health and safety studies of microorganisms.

Not applicable, as no confidentiality claims are asserted for information in the health or safety studies.

SUBSTANTIATION: 2. Production Volume P.V.

The information, marked '**P.V.**' in the text, is claimed as Confidential Business Information because it concerns details regarding the estimated yearly production with the rDNA microorganism.

Substantiation of the information, that is marked '**P.V.**' in the text, is given in the following, addressing both the general and the specific questions as required.

(c) (1). For what period of time is a claim of confidentiality being asserted? If the claim is to extend until a certain event or point in time, indicate that event or time period. Explain why the information should remain confidential until such point.

The information is claimed CBI for an unlimited period of time. Disclosure of this information would harm Novozymes' competitive position (see (c)(6)) below.

(c) (2). Briefly describe any physical or procedural restrictions within the company or institution relating to the use and storage of the information claimed as confidential. What other steps, if any, apply to use or further disclosure of the information?

The information is designated confidential and is only distributed internally to persons who have a documented need.

(c) (3). Has the information claimed as confidential been disclosed to individuals outside of the company or institution? Will it be disclosed to such persons in the future? If so, what restrictions, if any, apply to use or further disclosure of the information?

The information will only be disclosed to individuals outside the company only if there is a necessary part of obtaining approvals by authorities or business agreements. If the information is to be disclosed to such persons, it will only be done under a confidentiality claim or a secrecy agreement.

(c) (4). Does the information claimed as confidential appear, or is it referred to, in any of the following questions? If the answer is yes to any of these questions, indicate where the information appears and explain why it should nonetheless be treated as confidential. (i) Advertising or promotional materials for the microorganism or the resulting end product? (ii) Material safety data sheets or other similar materials for the microorganism or the resulting end product?
(iii) Professional or trade publications?
(iv) Any other media available to the public or to competitors?
(v) Patents?
(vi) Local, State, or Federal agency public files?

The information that is marked '**P.V.**' in the text does not appear nor is it referred to in any of the above mentioned documents.

(c) (5). Has EPA, another Federal agency, a Federal court, or a State made any confidentiality determination regarding the information claimed as confidential? If so, provide copies of such determinations.

No.

(c) (6). For each type of information claimed confidential, describe the harm to the company's or institution's competitive position that would result if this information were disclosed. Why would this harm be substantial? How could a competitor use such information? What is the causal connection between the disclosure and harm?

The information concerns details regarding the estimated yearly production of the rDNA microorganism and from this a yearly production of the protein products might be estimated. This information would be valuable to a competitor in calculating and evaluating important key figures, such as production economy and market size.

(c) (7). If EPA disclosed to the public the information claimed as confidential, how difficult would it be for the competitor to enter the market for the resulting product? Consider such constraints as capital and marketing cost, specialized technical expertise, or unusual processes.

See (c) (6) above.

(d) (1). Has the microorganism or method of production been patented in the U.S. or elsewhere? If so, why is confidentiality necessary?

Yes, the microorganism has been patented.

(d) (2). Does the microorganism leave the site of production or testing in a form which is accessible to the public or to competitors? What is the cost to a competitor, in time and money, to develop appropriate use conditions? What factors facilitate or impede product analysis?

The microorganism is removed from the final protein product.

(d) (3). For each additional type of information claimed as confidential, explain what harm would result from disclosure of each type of information if the identity of the microorganism were to remain confidential.

Not applicable.

(e). Health and safety studies of microorganisms.

Not applicable, as no confidentiality claims are asserted for information in the health or safety studies.

SUBSTANTIATION 3. Production Information P.I.

The information, marked **'P.I.'** in the text, is claimed as Confidential Business Information because it concerns details regarding the production information directly related to the process description.

Substantiation of the information, that is marked **P.I.** in the text, is given in the following, addressing both the general and the specific questions as required.

(c) (1) For what period of time is a claim of confidentiality being asserted? If the claim is to extend until a certain event or point in time, indicate that event or time period. Explain why the information should remain confidential until such point.

The information is claimed CBI for an unlimited period of time. Disclosure of this information would harm Novozymes' competitive position (see (c)(6)).

(c) (2) Briefly describe any physical or procedural restrictions within the company or institution relating to the use and storage of the information claimed as confidential. What other steps, if any, apply to use or further disclosure of the information?

The information is designated confidential and is only distributed internally to persons, who have a documented need.

(c) (3) Has the information claimed as confidential been disclosed to individuals outside of the company or institution? Will it be disclosed to such persons in the future? If so, what restrictions, if any, apply to use or further disclosure of the information?

The information will only be disclosed to individuals outside the company, if this is a necessary part of obtaining approvals by authorities or business agreements. If the information is to be disclosed to such persons, it will only be done under a confidentiality claim or a secrecy agreement.

(c) (4) Does the information claimed as confidential appear, or is it referred to, in any of the following questions? If the answer is yes to any of these questions, indicate where the information appears and explain why it should nonetheless be treated as confidential.
(i) Advertising or promotional materials for the microorganism or the resulting end product?
(ii) Material safety data sheets or other similar materials for the microorganism or the resulting end product?
(iii) Professional or trade publications?
(iv) Any other media available to the public or to competitors?
(v) Patents?
(vi) Local, State, or Federal agency public files?

The information, that is marked P.I. in the text, does not appear nor is it referred to in any of the above mentioned documents.

(c) (5) Has EPA, another Federal agency, a Federal court, or a State made any confidentiality determination regarding the information claimed as confidential? If so, provide copies of such determinations.

No.

(c) (6) For each type of information claimed confidential, describe the harm to the company's or institution's competitive position that would result if this information were disclosed. Why would this harm be substantial? How could a competitor use such information? What is the causal connection between the disclosure and harm?

The information concerns details regarding the production process. This information would be valuable to a competitor in evaluating important key processes including, but not limited to, confidential fermentation and recovery operations.

(c) (7) If EPA disclosed to the public the information claimed as confidential, how difficult would it be for the competitor to enter the market for the resulting product? Consider such constraints as capital and marketing cost, specialized technical expertise, or unusual processes.

See (c) (6).

(d) (1) Has the microorganism or method of production been patented in the U.S. or elsewhere? If so, why is confidentiality necessary?

Yes, the microorganism has been patented/patent pending.

(d) (2) Does the microorganism leave the site of production or testing in a form which is accessible to the public or to competitors? What is the cost to a competitor, in time and money, to develop appropriate use conditions? What factors facilitate or impede product analysis?

The microorganism is removed from the final enzyme product.

(d) (3) For each additional type of information claimed as confidential, explain what harm would result from disclosure of each type of information if the identity of the microorganism were to remain confidential.

Not applicable.

(e) Health and safety studies of microorganisms.

Not applicable, as no confidentiality claims are asserted for information in the health or safety studies.

1. SUBMITTER IDENTIFICATION

Submitter

The company which is submitting the Microbial Commercial Activities Notification (MCAN) manufactures biotechnologically derived products. The submitter of this MCAN is:

Novozymes North America, Inc.
77 Perry Chapel Church Road
Box 576
Franklinton, NC 27525

Principal Technical Contact

The technical contact for this MCAN is:

Denise Bernstein, M.S., M.B.A
Staff Specialist
Novozymes North America, Inc.
77 Perry Chapel Church Road
Box 576
Franklinton, NC 27525

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I. INTRODUCTION

The fungus *Trichoderma reesei* production strain [redacted] contains a gene from [redacted], to produce cellulose-degrading enzymes to be used for ethanol production. [redacted], in addition to the proteins produced by the *Trichoderma reesei* host itself (cellulases), are used primarily in biomass conversion of [redacted]. M.I.

II. MICROORGANISM IDENTITY INFORMATION

1. Recipient Microorganism (Host Strain)

1.1 Taxonomy

The host strain, [redacted] is *Trichoderma reesei* derived from strain [redacted]. M.I.

The taxonomic characteristics of the host strain of *Trichoderma reesei* [redacted], are the following:

Name:	*Trichoderma reesei*
Class:	Sordariomycetes
Order:	Hypocreales
Genus:	*Trichoderma*
Species:	*reesei*

Reference: Kuhls K., Lieckfeldt E., Samuels G.J., Kovacs, Meyer W., Petrini O., Gams W., Borner T. & Kubicek C.P. 1996. Molecular evidence that the asexual industrial fungus *Trichoderma reesei* is a clonal derivative of the ascomycete *Hypocrea jecorina*. *Proc. Natl. Acad. Sci. U.S.A.* 93 (15): 7755-7760 (1).

1.2 Pathogenic and Physiological Traits

Trichoderma reesei is a filamentous fungus that reproduces strictly asexually (imperfect fungus). On the basis of DNA analysis, *Trichoderma reesei* has been classified as the asexual form of the ascomycete *Hypocrea jecorina* (1).

Trichoderma. reesei, H. jecorina or previous names used for the strain have not been listed in Berufgsnossenschaft der Chemischen Industrie Merkblatt B 007/Pilze (nr 8/2002) as being Biological Risk class 2 or higher. It is classified as a Class 1 organism according to the NIH guidelines. Thus, both *Trichoderma* and *Hypocrea* species are regarded as Biological Risk Class 1 organisms and are not considered pathogenic to humans. Extracellular cellulases are produced by many bacteria and fungi normally found in environments rich in decaying plant material. An analytical report on the 'Metabolite potential of *Trichoderma. reesei*' concludes that Novozymes' strains of *Trichoderma. reesei* do not produce secondary metabolites nor mycotoxins (Appendix A -- Analytic Report, Center for Microbial Biotechnology BioCentrum – DTU, Technical University of Denmark, September 2004).

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1.3 Prior Reports of Extended History of Safe Industrial Use

Trichoderma reesei has a long history of safe use in industrial-scale enzyme production (2-5). Historically, cellulases produced by *Trichoderma reesei* are used in food, animal feed, pharmaceutical, textile, pulp and paper applications (2). According to Hjortkjaer, industrial cellulases produced by fungi including *Trichoderma reesei* "[have] a long history of safe use in the production of food and as digestive aids without having given any evidence of possible toxicity" (5, see p. 62). In addition, a pectin lyase enzyme produced by *Trichoderma reesei* was determined to be a GRAS (Generally Recognized As Safe) substance by qualified experts and has been determined to be safe for food uses (GRAS notice 000032 letter, 20- April-2000; http://www.cfsan.fda.gov/~rdb/opa-g032.html).

Further, numerous notifications using *Trichoderma reesei* as host organism have been reviewed and found to be acceptable by EPA (see MCANs listed under EPA's Biotechnology Program Under TSCA Notifications, FY98 to Present; www.epa.gov/biotech_rule/pubs/submiss.htm), i.e., J03-0001, J03-0002, J04-0001, J04-0005, J05-0001, J05-0002, J06-0001, J07-0001, and J09-0002).

Also, the current *Trichoderma* host has been used as a non-genetically modified production strain by Novozymes for commercial production of cellulase and xylanase products (3) as well as a genetically modified production strain for protein preparations (see J07-0001 & J09-0002). Nevalainen *et al.*, concluded that recombinant "techniques have been used to improve the industrial production strains of *Trichoderma reesei* and, in addition, considerable experience of safe use of recombinant *Trichoderma reesei* strains in industrial scale has accumulated. Thus, *Trichoderma reesei* can be generally considered not only a safe production organism of its natural enzymes, but also a safe host for other harmless gene products." (2, abstract).

As stated in section 1.2, an analytical report on the 'Metabolite potential of *Trichoderma. reesei*' concludes that Novozymes' strains of *Trichoderma. reesei* do not produce secondary metabolites nor mycotoxins (Appendix A -- Analytic Report, Center for Microbial Biotechnology BioCentrum – DTU, Technical University of Denmark, September 2004).

Based on the foregoing information, it is concluded that the current *Trichoderma reesei* organism can be considered a safe host organism for expressing industrial proteins and can be classified as a non-pathogenic and non-toxigenic microorganism as well.

1.4 Development of the Host Strain

[redacted], was genetically modified from the wild-type strain, [redacted], using [redacted].

[redacted] M.I.

↓ [redacted]

[redacted]

↓ [redacted]

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[redacted] M.I.

↓ [redacted]]

[[redacted]]

2. Donor Organisms

2.1 Donor organism for the Expressed Gene – [redacted]. M.I.

[redacted]

2.2 Pathogenic & Physiological Traits and Safe Use of Protein

The genetically modified *Trichoderma reesei* strain contains a gene from [redacted] M.I. [redacted] is classified as a Risk group 1 organism according to NIH guidelines. The genetically modified organism is classified as a Risk group 1 organism.

2.3 Donor for the promoter

The promoter is from *Trichoderma reesei* strain [redacted]. M.I.

2.4 Donor for the terminator

The transcriptional terminator is from *Trichoderma reesei* strain [redacted]. M.I.

3. Recombinant Microorganism – Identification of the Production Strain

The recombinant microorganism is Trichoderma reesei [redacted]. M.I.

4. Construction of Recombinant Microorganism

4.1 [redacted] gene. M.I.

The [redacted] encodes a secreted protein that [redacted]. The gene was inserted between the *Trichoderma reesei cbh1* promoter and *cbh1* terminator.

4.2 Structure of the expression vector

4.2.1 [redacted]. M.I.

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M.I.

Figure 1. Plasmid map of [redacted] expression plasmid.

M.I.

[Position (bp)	Size (bp)	Element	Origin

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4.3 Construct Information

4.3.1 [redacted] **M.I.**

Description of the Construction of the [redacted].

M.I.

[redacted] Standard laboratory procedures were used to amplify a DNA fragment. The reaction products were isolated on a 1.0% agarose gel using TAE buffer where a [redacted] product band was excised from the gel and purified using a commercial extraction kit according to the manufacturer's instructions.

The fragment was then cloned into the [redacted]using an Infusion Cloning Kit. The vector was digested with restriction enzymes using conditions specified by the manufacturer. The fragment was purified by gel electrophoresis and gel purification. The gene fragment and the digested vector were ligated together in a reaction resulting in the expression plasmid [redacted] in which transcription of the [redacted] gene was under the control of the *T. reesei* cbh1 promoter. [redacted].

[redacted]

4.4 Construction of Recombinant Production Organism M.I.

[redacted]

[redacted]

4.4 Stability of the Construction and Genetic Transfer Capability

The recombinant production strain is expected to be stable upon prolonged growth. The inserts are chromosomally integrated and, as a result, they are poorly transferred to other organisms.

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4.6 Antibiotic Resistance Gene

The strain does not contain any antibiotic resistance genes.

5. Classification of the Production Strain

As discussed in sections 1.2 and 1.3, *Trichoderma reesei* is generally regarded as a non-pathogenic fungus that is widely distributed in nature. It is classified as a Class 1 organism according to the NIH guidelines.

The host organism is non-pathogenic and non-toxigenic and the introduced genetic materials do not encode any known harmful or toxic substances. As a result, the genetically modified *T. reesei* production strain [redacted] is considered non-pathogenic and non-toxigenic and safe for its intended use. **M.I.**

The production strain complies with the OECD (Organization for Economic Co-operation and Development) criteria for GILSP (Good Industrial Large Scale Practice) microorganisms.

6. Phenotypic and Ecological Characteristics

Trichoderma reesei is widely distributed in nature (see sections II & VII).

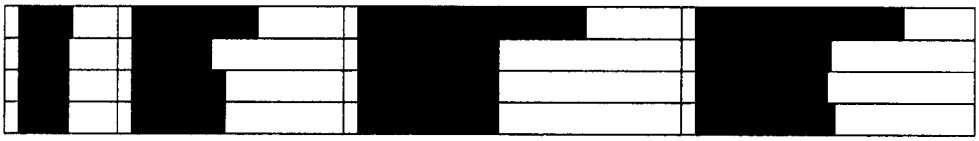
III. BYPRODUCTS

Solid and liquid wastes generated from the production of cellulose-degrading enzymes are sterilized by a combination of filtration and chemical and physical treatments to ensure that viable cells of the production microorganism are not present in the material released to the environment.

IV. TOTAL PRODUCTION VOLUME

The modified microorganism, which is the subject of this MCAN, is used to produce an enhanced cellulolytic protein preparation. The estimated microbial biomass to be produced annually during the first 3 years of production for years 2009-2011 is listed below in Table 1.

Table 1. Estimated Total Microbial Biomass Annual Production for 2009-2011 **P.V.**



V. USE INFORMATION

1. Microbial Substance

Trichoderma reesei, which is the subject of this MCAN, will be used for the biosynthesis of cellulose-degrading enzymes to be used for ethanol production. The production microorganism will be removed from the commercially sold enzyme product. The biomass produced during the fermentation is removed from the recovery process stream, inactivated, and discarded.

2. Protein Preparation from the Recombinant Strain

The current use is to produce cellulose-degrading enzymes to be used for ethanol production. The enzyme preparation will work synergistically with other enzymes.

VI. WORKER EXPOSURE AND ENVIRONMENTAL RELEASE

1. Site Identification

The modified *Trichoderma reesei* microorganism will be used in a manufacturing process at the following location:

Novozymes North America, Inc.
77 Perry Chapel Road
Franklinton, North Carolina 27525.

2. Process Description

The manufacturing plant, discussed above in section VI.1., produces other food grade and industrial-grade proteins using processes that have varied only slightly for over 25 years. During this time, the company has observed procedures based on physical and biological containment and appropriate work practices designed to minimize both the environmental and occupational exposure to the microorganisms used in the manufacturing processes.

The manufacturing process that utilizes the microorganism is a two-step process involving fermentation and recovery steps. The fermentation process comprises 3 main operations: 1) laboratory propagation of the culture; 2) seed or inoculum fermentation; and 3) the main fermentation. These process steps and recovery processes are described in more detail below and an schematic outline of a typical fermentation process flow and a recovery process flow are provided in the published literature (Appendix B -- Hjort, C.M., 'Production of Food Additives using Filamentous Fungi' in Genetically Engineered Food: Methods and Detection. Edited by Knut J. Heller, Wiley-VCH Verlag GmbH & Co, 2003, p.97).

2.1 Fermentation

The process used to grow the modified organism and produced the proteins of interest is submerged (or deep tank), aerobic, pure-culture fermentation. The process, except for the preparation of the initial culture, is carried out in sealed vessels carefully designed to prevent both the release of the production organism and the entry of other microorganisms.
The modified organism is an aerobe, an organism requiring oxygen for life and growth. In this respect, the modified organism is similar to the majority of microorganisms used by industry to

produce proteins including enzymes, antibiotics as well as biopolymers and other substances that are manufactured by fermentation processes. In order to supply the required oxygen and prevent the introduction of other microorganisms, fermenters used for this type of fermentation are closed pressure vessels equipped with top mounted agitators with sterilizable seals where the agitators enter the vessel, (e.g., double mechanical seals), bottom-mounted air sparger rings to supply and disperse sterile air, and cooling/heating coils for temperature control. During the sterilization the vessels are usually pressurized to 1-2 bars and during fermentation, a pressure greater than atmospheric is usually maintained. The seed and main fermenters used in the remaining two steps in the fermentation process are of this type.

The ingredients used to prepare the nutrient solutions (broths) in which the organism is grown included various carbon and nitrogen sources and other inorganic salts and trace metals (see P94-1475). These ingredients are of suitable quality, free of harmful or deleterious substances and free of substances that would inhibit microbial growth or production of the desired proteins or polymers. Each batch of each substance is sampled and tested by the Quality Control Department to ensure the product is in conformance with the desired specifications.

2.2 Laboratory Propagation

A pure culture of the modified organism, which has been maintained as a lyophile or by storage under liquid nitrogen or other standard technique, is aseptically transferred to a flask containing a sterile nutrient agar prepared from various approved ingredients discussed above.

The techniques used to transfer the organism are designed to prevent both the introduction of other organisms into the flask and the release of the modified organism.

The organism is grown until the desired colony formation and density have been obtained. During this time period, the rate of colony formation and the growth, size, and the appearance of the colonies are examined to ensure that a pure culture has been obtained.

When the desired growth level has been reached, the colonies are transferred to a second flask by washing the agar with sterile water from the second flask which had been connected to the agar-containing flask prior to preparation of the initial sterile agar to give a closed system. This process yields a culture suspension that will be used in the next process step.

After transfer of this suspension to the next process step, the seed fermentation, the residue in the flask is examined for foreign microorganisms by plating on standard agar and other tests if desired.

2.3 Seed Fermentation

Prior to preparation of the medium, the seed fermenter is cleaned with a caustic solution, rinsed thoroughly with potable water, then steam sterilized and cooled. The culture medium is prepared from ingredients drawn from the above list, mixed thoroughly and sterilized by heating to 121°C.

After the sterile medium is cooled to the desired temperature, the culture suspension obtained from the laboratory step is transferred aseptically to the sterile medium.

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In this step, growth of the organism is continued until the desired biomass is obtained. During growth, parameters such as temperature, aeration rate, and pH are monitored and samples are withdrawn periodically and examined microscopically to ensure culture purity.

2.4 Main Fermentation

The main fermenter is prepared by cleaning with a solution of caustic, rinsing with potable water and steam sterilizing while empty. The growth medium is prepared in a mixing tank using potable water and ingredients drawn from the acceptable list of ingredients. The medium is transferred to the main fermenter and sterilized by heating the mixture under pressure to a temperature greater than 121°C.

Nutrient feed solutions, if required, are prepared and sterilized in closed mixing tanks, using approved ingredients. After preparation of the sterile nutrient solutions is completed, the contents of the seed fermenter are aseptically transferred to the main fermenter where growth of the organism continues. The desired proteins are produced by the organism during this step. During this step, sterile nutrient solutions can be added to the fermenter and portions of the medium can be withdrawn from the fermenter.

The growth of the organism and the production of the proteins of interest are closely monitored by continuous measurement of parameters such as temperature, pH, aeration rate, dissolved oxygen, etc, and performing tests and analyses such as protein concentration, culture purity, nutrient levels, etc., on samples that are withdrawn from the fermenter at predetermined levels.

The procedure used to obtain samples from the fermenter is designed to prevent microbial contamination of the fermenter contents and sample, and minimize environmental release of the producing organism as described in the environmental release and disposal section.

The fermentation process is continued until laboratory test data show that the maximum protein production has been obtained or the rate of protein production falls below a predetermined value. When this condition is reached, the fermentation is stopped.

The sparge rate of the fermenter is typically one volume of air/volume of medium/minute. The rate would reach a maximum of [redacted] m^3/minute depending on the volume of the fermenter which typically ranges from [redacted] m^3. P.I. P.I.

2.5 Recovery

The recovery process is designed to separate the desired protein from the biomass and to purify, concentrate, and stabilize the protein preparation.

P.I.

[redacted]

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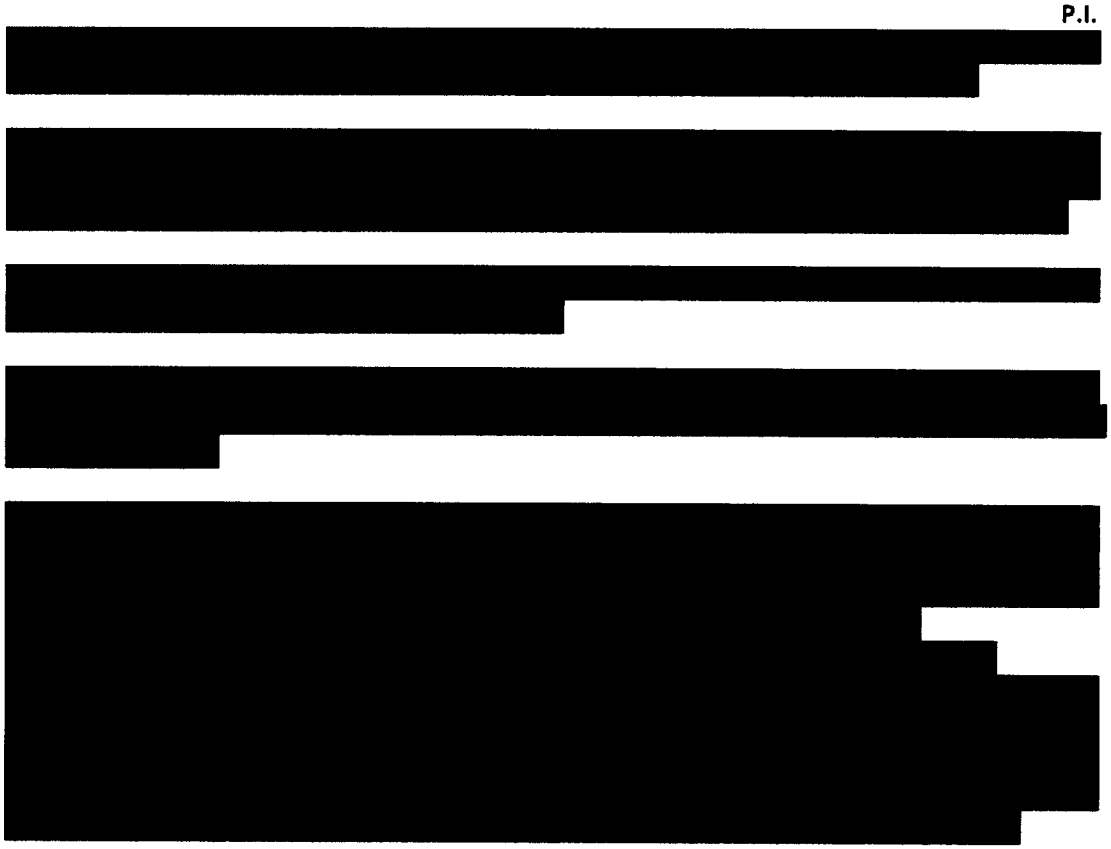
P.I.



The concentrated protein preparation from the steps described above is formulated at the production facility into commercial protein preparations by adding diluents such as sodium chloride solution, propylene glycol or other suitable substances and, if necessary, additional preservatives such as those mentioned above. During this portion of the process, the enzyme activity of the preparation is standardized to the desired level.

3. Worker Exposure

3.1 Company Overview

The company management is dedicated to providing a safe work place. For over 25 years, workers have safely handled microorganisms at this site. Only non-pathogenic and non-toxigenic microorganisms are used at the plant. Worker exposure to microorganisms and proteins are kept at a minimum. This is accomplished by a broad range of established Standard Operating Procedures including those that ensure minimum exposure to all microorganisms that are or may be used in the production processes.

Novozymes is dedicated to providing a safe work environment. A broad range of actions are in place including those that ensure minimum exposure to all microorganisms that are used in the production processes. These actions include the following:

- Use of nonpathogenic, non-toxicogenic microorganisms in all fermentation processes. As stated in section II of the MCAN, the modified microorganism meets these criteria.

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- Design and selection of process equipment that provide a level of containment that is appropriate for the production microorganisms - Good Industrial Large Scale Practice (GILSP).
- A comprehensive, plant-wide safety program.
- Installation of appropriate engineering controls.
- Procedures for routinely monitoring the performance of the installed engineering controls.
- Developing procedures and work practices that minimize exposure.
- Training in the proper use of the process equipment and safe work practice.
- Providing personal protective equipment for use where necessary and training in use of this equipment.
- An employee health education and monitoring program. A full-time occupational health and safety manager and nurse are on site to conduct training, perform exposure monitoring, and monitor these programs.
- Personnel training in safe handling of all chemicals as required by OSHA regulations.
- Additional training required to safely perform special operations.

Due to the nature of the manufacturing process, the possibility of occupational exposure to the modified microorganism that is the subject of this MCAN is limited to personnel involved in or with the early steps in the process; specifically the laboratory propagation and testing, the fermentation steps and the first steps in the recovery process during which the modified microorganism is removed and inactivated. The final product, the commercial protein preparation product, has been processed to remove the entire modified microorganism.

3.2 Containment

Novozymes has performed a careful evaluation of the modified microorganism and the planned manufacturing process using information that has been included in this MCAN and information on the historical use of the host organism (unmodified) as well as other historical information from other, similar fermentation processes (see section 1.c of the MCAN). This evaluation has led to the conclusion that the process will be performed under conditions that, as a minimum, will be equivalent to the containment level recommended in the original 1986 report titled "Recombinant DNA Safety Considerations" published by the Organization of Economic Cooperation and Development (OECD) (Paris). The recommendations set forth by OCED are reinforced by the National Institutes of Health's original Guidelines for Research Involving Recombinant DNA Molecules by the Recombinant DNA Advisory Committee and the Office of Recombinant DNA Activities and subsequent amendments located on its website (http://www4.od.nih.gov/oba/rac/guidelines/guidelines.html). This level of containment and regular sampling ensure that employee exposure and unintentional release to the environment are below the level that could raise safety concerns.

3.3 Engineering Controls

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The processes described above discuss a sterilizable piping system through which culture liquids are transferred, and the closed processing tanks form a nearly completely closed system. The [redacted] are completely enclosed with stainless steel hoods equipped with local exhaust that minimizes occupational exposure to the incidental release of microorganisms during the filtration steps. The hoods contain curtains or doors that can be opened to check the operation of the [filter] but still allow for containment of any aerosols present. Filtration of a protein preparation as set forth in this MCAN requires between [redacted] and

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P.I.

operators are present in the area around the filters for approximately 20 to 30% of this time, not counting the time required for [redacted] during which no protein is present. These filtration systems and procedures are used for all fermentation processes in which the producing organism is removed by filtration. Many of the process steps are controlled by computer systems and other automated control systems that are located in a control room that is isolated from the manufacturing area. These systems provide an additional form of engineering control in that the use of these systems decreases direct contact of operating personnel with the products being manufactured. Air monitoring in the area of the [redacted] and other process equipment includes sampling of both proteins and production microorganism levels. Sampling is conducted on a daily basis in areas that contain equipment with the potential to generate aerosols such as drum filters and ultra-filtration. High volume industrial hygiene samplers are used to measure enzyme levels in the air. Samples are collected on 15cm glass fiber filters and are analyzed through the use of activity-based or ELISA methods. Airborne enzyme results are compared against published exposure limits and internal standards to prevent the sensitization and possible development of allergy to the enzyme proteins. Samples that exceed the internal exposure limit of 45 ng/m3 result in follow-up measures which include cleaning, additional air monitoring, and the use of personal protective equipment such as respirators until clearance samples have been obtained.

3.4 Procedure and Work Practices

Effective control of occupational exposure is a goal that requires the active participation of all company personnel in the development and daily use of a combination of appropriate operating procedures and work practices, and the use of appropriate personnel protective equipment where such equipment is required.

To help achieve this goal, production departments conduct routine safety inspections of their work areas, perform monthly training and hold departmental safety meetings involving both supervisory personnel and operators. A larger cross-functional safety committee meets monthly to review accidents and near-misses as well as topics that affect the larger organization. Training is held regularly for current employees and to train new employees. In addition, another committee oversees conformance with current Good Manufacturing Practices (cGMP) regulations. The committee meets generally once of a month to review conformance with these regulations and discuss any actions needed in this area.

3.5 Health Program

Occupational health and wellness is an important part of the company's philosophy. Annual health screenings are conducted for all production, laboratory, and maintenance personnel. These examinations include audiometric testing, pulmonary function testing, respiratory fit testing and a blood sample test to determine the levels of antibodies to the specific enzyme proteins handled at the Franklinton, NC location. An annual history form is also completed by each employee as part of the examination. The information obtained from the screening and history is reviewed by the company physician and occupational health nurse. The test information and any comments received from the reviewing physician are reviewed with each employee and a summary of the test results is given to each employee.

The Company also has a global Medical Center headed by an Occupational Physician to provide support and guidance for occupational health. Safety & Health personnel from the North Carolina location interact frequently with their counterparts in other global sites. To work towards the

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goal of eliminating enzyme allergy in the workplace, Novozymes has implemented a program called Zero Enzyme Allergies (ZEAL) that includes occupational health screening as well as emphasis on engineering controls, work practices, and personal protective equipment (PPE). Performance metrics are reported on and tracked globally.

3.6 Employee Exposure

As mentioned previously, the manufacturing process is performed in a predominantly closed, highly automated system which limits the potential exposure of employees to the modified organism. Table 2 depicts the Average Workers per Facility and Process.

Table 2. Average Workers per Facility and Process **P.I.**

[a] Microbiology personnel provide partial coverage to night shift
[b] Production shifts are 12 hour rotating

4. Information on Release of the Production Organism

An environmental monitoring system is in place to quarterly test for the presence of GMOs in solid and liquid wastestreams. This monitoring system is designed to exceed the compliance requirements as set forth in Part 725 - the TSCA Reporting Requirements and Review Process for Microorganisms. The facility also has control equipment that reduces the population of microoganisms in air emissions.

5. Transport of the Production Organism

As indicated previously, the production organism will be used for production of cellulose-degrading protein preparations at only one site in the U.S. and the production microorganism is removed from the commercial product. In general, the production organism will not be transported unless for disposal purposes. Treated wastewater, which is in compliance with the regulations as discussed in section VI.4, may occasionally be transported to municipal wastewater treatment plants.

6. Process Waste Disposal

6.1 Permits

Typically, Novozymes sprays treated wastewater on privately-owned land. The company also discharges a portion of treated wastewater to a publicly-owned waste treatment plant under permit. The biomass produced during fermentation is an intermediate product that is removed from the process stream, inactivated, and discarded as described below. The use of the inactivated biomass as fertilizer is licensed by the North Carolina State Department of Agriculture.

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The current permits are: P.I.

[redacted]

P.I.

The recovery process at Novozymes described above generates a "solid" waste, an [redacted] and spent biomass from the [redacted] and other filtration steps employing [redacted], and a liquid waste from the [redacted] and the wash water used in equipment cleaning operations. Disposal of these process wastes are carried out as described below.

6.2 Solid Wastes

P.I.

[redacted] At the end of each [redacted] and filtration is continued until the material in the trough has been diluted substantially, as indicated by [redacted] The remaining contents of the trough are transferred to the sludge storage tanks. [redacted]. The storage tanks are sampled and tested quarterly to ensure that the slurry does not contain any viable production organism. A portion of the combined biomass is dewatered through a decanter centrifuge. The concentrate from the centrifuge is routed to the wastewater treatment system. Calcium hydroxide is blended with the cake as it drops from the centrifuge to raise the pH above 12.0. The cake is [redacted]. The company also has the capability under permit to land-fill or land-apply the cake as a dry material.

P.I.

Final disposal is by land application to approximately [redacted] acres of agricultural fields owned by the company and neighboring landowners. The diluted sludge is surface applied to the soil. The nitrogen and other plant nutrients in the waste are used as fertilizer. This disposal operation is carried out under permit [redacted], granted by the State of North Carolina Department of Environment and Natural Resources, under the applicable EPA regulations governing the disposal

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of solid wastes. It is also a registered fertilizer by the North Carolina Department of Agriculture. This method of solid waste disposal has been used for 26 years.

Occasionally filter pads need to be placed in a landfill. In this circumstance, these filter pads are first treated with caustic (potassium hydroxide) and then sent to a municipal solid waster type landfill (Brunswick Waste Management Landfill, 107 Mallard Crossing Rd., Lawrenceville, VA 23868). GMM testing is conducted prior to any release of filter pads to the landfill.

6.3 Liquid wastes

P.I.

Liquid waste is generated in the form of wastewater from the [redacted], and spent wash water and cleaning solutions from the process tanks and equipment. The cleaning operations are conducted with [redacted] The production organism has not been detected in the collection sump. The wastewater in the sump is pumped through a primary clarifier to an activated sludge treatment system owned and operated by Novozymes and located on Novozymes property. The activated sludge system is designed to reduce the organic carbon and nitrogen levels in the wastewater, and to prevent emission of odors. A final clarifier separates wastewater biomass from liquid by gravity separation. The primary clarifier and waste activated sludges are combined with the spent biomass at the buffer tank.

P.I.

The treated wastewater is collected in two lagoons for subsequent land application by spray irrigation, under permit [redacted] granted by the State of North Carolina Department of Environment and Natural Resources, under the applicable EPA regulations governing the disposal of wastewater. The Company also discharges a portion of the wastewater to local municipal wastewater treatment systems through a pipeline. In addition, a "pump and hall" permit can be obtained, if needed, which is granted with the permission of the municipality and the State of North Carolina Department of Environment and Natural Resources, under the applicable EPA regulations governing the disposal of wastewater. The lagoons are sampled quarterly and tested for the presence of the organisms used for enzyme production.

Novozymes discharges non-production waste (domestic sewage) directly to the Franklin County Wastewater Treatment Plant, and Discharges cooling tower water to surface water through permit [redacted]. Both of these waste water streams have been determined not to contain GMM based on sampling and our process knowledge.

6.4 Exhaust Air

P.I.

The production organism requires [redacted]. Exhaust air from all fermenters is collected in a plenum which is kept under a slight negative pressure. This air stream passes through a cyclone separator designed to remove entrained droplets and mist. Any collected liquid and solids is sent to the wastewater treatment system or spent biomass treatment system described above. The off-gas is then discharged to the atmosphere through the fermenter exhaust stack. [redacted]

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Although, air sampling was conducted in the past, the company has instituted a worldwide initiative to conduct soil sampling in lieu of direct air sampling. This policy change is a result of a 1998 EU Directive (No. 98/81, October 26, 1998) and an agreement between the Danish authorities and Novozymes that monitoring soil samples for growth of GMMs is a better way of determining environmental impact than actual monitoring air samples. As a result, Novozymes developed a worldwide soil/vegetation sampling program. Soil/vegetation sampling is viewed as a better means of controlling emissions and is a more reliable measurement of the impact of GMMs on the environment than air sampling.

P.I.

The exhaust air from the [redacted] is vented to the roof of the production facility.

VII. HEALTH AND ENVIRONMENTAL EFFECTS DATA

1. Environmental Testing and Monitoring Program

Novozymes has established a Genetically Modified Microorganism (GMM) monitoring program including the standardizing operating procedures for the Determination of GMO Environmental Action Levels Using Fermentation Populations, and Detection of Production Strains in Environmental Samples. The GMM monitoring program consists of sampling in four areas: measurement in waste water, biomass, filter pads for landfill (if applicable), and a retrieval program of soil samples. Typically, 2 measurements of bacterial and fungal production strains are tested in waste water, biomass, and filter pads for landfill are tested quarterly. The soil retrieval program is tested one time per year near the end of the growing season at five locations at the NZNA site. The purpose of the soil retrieval program is to see if GMMs are able to establish and survive in the environment outside the closed systems. Based on monitoring samplings, environmental release is negligible.

2. Environmental Programs and Initiatives

(a) EPA National Performance Track Program

Novozymes North America, Inc. has been a member of the EPA Performance Track Program since 2001. All of our goals have been met or exceeded in the first round, which resulted in significant reduction in water, solid waste, and volume of residual biomass land-applied along with a significant increase in recycling (https://yosemite.epa.gov/opei/ptrack.nsf/vAPRViewPrintView/EFF9A6F3772E300D85256E8200739D26). The company expects to meet or exceed all of its goals for the second round of the program (https://yosemite.epa.gov/opei/ptrack.nsf/vRenewalViewPrintView/225B5BCE36D5328185256E9700642E0A).

(b) State of North Carolina's Environmental Stewardship Initiative Program

Novozymes North America, Inc. was selected as the State of North Carolina's first Environmental Steward in its Environmental Stewardship Initiative program (http://www.p2pays.org/esi/; http://www.p2pays.org/news/press_releases/022703.asp).

3. Conclusions

Along with bacteria and other fungi, *Trichoderma reesei* is widely distributed in nature especially among decaying matter. A non-genetically modified *T. reesei* has been used safely for the production of cellulases at this site since 1999. Based on a review of the literature, *Trichoderma reesei* has not been known to be associated with human or veterinary pathogenicity. The exception would be the possibility of an opportunistic infection in severely immunocomprised or debilitated individuals. Further, there are no known reports to indicate the formation of toxins from this species or strains of *Trichoderma reesei* (see Appendix A).

Existing classification of *Trichoderma reesei* as a Biological Risk Class I microorganism further substantiates that this microorganism is low risk and is not a potentially dangerous organism. There is one laboratory-contained soil study in the literature showing preliminary results that *Trichoderma reesei* may persist in soil for 2 seasons (7).

In conclusion, the production organism is not part of the final protein preparation product sold to customers, the microorganism is handled in a contained environment at the plant site, the microorganism is inactivated prior to release to the environment and subsequently very little, if any, viable micoorganism is expected to enter the environment. Therefore, based on this information, *Trichoderma reesei* does not present an unreasonable risk to the environment and to humans, (apart from the possibility of an opportunistic infection in severely immunocomprised or debilitated individuals).

VIII. LIST OF REFERENCES

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IX. Appendices

Appendix A. Analytical Report – Metabolite potential of *Trichoderma reesei*

Appendix B. Hjort, C.M., 'Production of Food Additives using Filamentous Fungi' in Genetically Engineered Food: Methods and Detection. Edited by Knut J. Heller, Wiley-VCH Verlag GmbH & Co, 2003, p.97

4
Production of Food Additives using Filamentous Fungi

Carsten M. Hjort

4.1
Filamentous Fungi in Food Production

Fungi are eukaryotic microorganisms that have been used either as foods or for the manufacture of food for more than a thousand years. The fungal kingdom consists of yeasts that are unicellular organisms, and also of filamentous fungi that are multicellular organisms with the cells organized in chains known as hyphae. The hyphae can be branched to greater or lesser degrees. Some fungi are dimorphic, which means that they have both unicellular and filamentous growth stages.

Yeasts have been used extensively for food production. The yeast *Saccharomyces cerevisiae* (baker's yeast) is used in baking, in brewing and in winemaking. In all of these applications the ability to ferment glucose to ethanol and carbon dioxide is the key feature.

Following the advent of recombinant production technology, a variety of yeasts have been used for the production of enzymes and metabolites, and recombinant expression systems suitable for large-scale production have now been developed for *S. cerevisiae*, *Schizosaccharomyces pombe*, *Pichia pastoris*, *Pichia methanolica*, *Hansenula polymorpha*, *Kluyveromyces lactis*, *Yarrowia lipolytica*, and other species [1, 2].

In the production of food enzymes however, filamentous fungi are much more broadly employed than yeast systems, and have been used in this role for a very long time. In some cases, the fungus is the food itself (an example is mushroom, *Agaricus bisporus*), but more recently the ascomycete *Fusarium venenatum* was developed as a single cell protein food source marketed under the tradename Quorn^{TM} by the company Marlow Foods [3]. In a few cases, the fungus is actually an ingredient of the food, as in the case of cheese, where various species of *Penicillium* (e. g., *P. roqueforti* and *P. carmemberti*) form part of the cheese product. The oldest examples of using filamentous fungi in food production are in the fermentation of food. In Japan, the filamentous fungi *Aspergillus oryzae* (Fig. 4.1), *Aspergillus sake* and related species have been used for fermenting sake, shoyu, and

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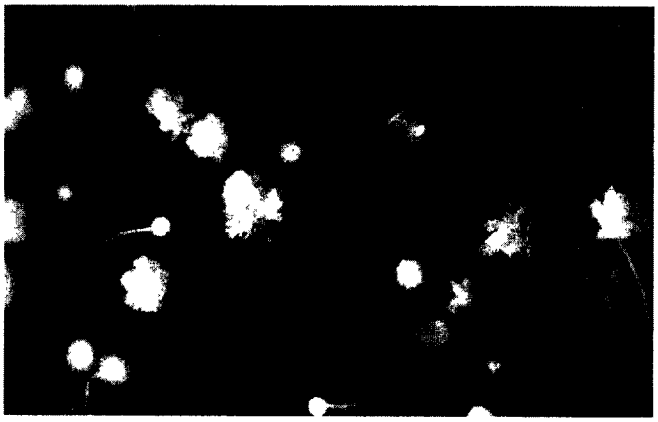

Figure 4.1 *Aspergillus oryzae* agar culture. A strain of *A. oryzae* was grown on an agar plate;, various stages of sporulating phialides can be seen in the photomicrograph.

miso. In these processes the fungus ferments sugar to alcohol, but an equally important point is that the fungus secretes enzymes such as proteases and amylases that modify the raw material into the desired product.

4.1.1 Industrial Applications

The potential for the food industry of the enzyme complex produced by *A. oryzae* was acknowledged late in the nineteenth century when, in 1894, the Japanese-American enzyme pioneer Jockichi Takamine first manufactured an enzyme complex from *A. oryzae* that was sold under the tradename Takadiastase™ [4]. Later, during the 1950s the submerged fermentation industry was developed in the United States, with *A. oryzae* being used to produce an array of enzyme products in submerged fermentation, but still with the emphasis on proteases and amylases. Other *Aspergillus* species such as the black aspergilli, *Aspergillus niger*, *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus aculeatus* and *Aspergillus japonicus*, were also used mainly to produce the enzyme glucoamylase [5].

Glucoamylase formed the basis for the enzyme revolution in the starch industry. Traditionally, starch was acid-hydrolyzed to glucose using hydrochloric acid, followed by neutralization. During this process several by-products were formed, and heavy salt formation resulted from the neutralization step. By introducing enzymatic hydrolysis, the by-product formation was avoided and so a product of a better quality could be manufactured at a lower price.

A. niger was also used in the production of primary metabolites in bulk amounts, for example citric acid [5]. Hence, fermentation process development was carried out both for enzyme and metabolite production. The production strains were also dramatically improved by mutagenic manipulation of the strains using either chemicals or radiation, followed by yield screening.

Even though major improvements have been achieved using classical strain improvement and fermentation optimization, recombinant DNA technology has revolutionized microbial enzyme production, and for three main reasons:

1. It is possible to produce enzymes isolated from virtually any organism, in good yields, in a highly developed and safe expression system. The accumulated toxicity and safety studies for products created in these systems provides excellent documentation for their safety. In this way, several different enzyme products can be generated using the same equipment, and essentially the same process.
2. The host organism (the organism into which the foreign DNA is transformed) can be modified to suit the quality specifications of the enzyme product. These modifications include removal of unwanted side activities that might result in a product purity that cannot be obtained using nonrecombinant methods. The fermentation yields of the enzyme can often also be increased several fold compared with yields obtained from a donor organism.
3. Using recombinant DNA technology, it is possible to produce genetically engineered enzymes. Such enzymes may have one or more amino acids substituted for other amino acids, or they may be hybrids between two or more enzyme genes. By using genetic engineering, it is possible to obtain enzymes with substantially improved properties.

At the dawn of the age of recombinant technology production, one of the first – and one of the most critical – choices that had to be made was that of expression systems. The extensive experience acquired with *Aspergillus* sp. made them clear candidates as host organisms: first, they were known to be able to produce huge amounts of extracellular protein; second, they were well-suited for production in submerged cultures in stainless steel tanks; and third, mutants that were even better adapted to this production environment had been selected. Due to the long-term use of *A. oryzae* [6] and *A. niger* [7] as production organisms for food enzymes, materials produced by these organisms have for several years been recognized by the FDA as GRAS (Generally Regarded As Safe).

Although *Aspergillus* species such as *A. oryzae* [8], *A. niger* and *A. awamori* [9] are dominant in enzyme production, other fungal systems have also been successfully developed for this purpose. *Trichoderma reesei* (also known as *Trichoderma longibrachiatum*) is a wood-degrading fungus that first attracted attention due to its ability to produce huge amounts of cellulases and hemicellulases. *T. reesei* proved to be suitable for submerged fermentation and was subsequently developed as an expression system [10].

More recently, a new fungal expression system – the *Fusarium venenatum* expression system – has been developed [11]. This fungus is used for single cell protein production (see above) and is well-suited for submerged fermentation, largely because the fermentation broth is easily aerated as a consequence of its rheological properties. The long-term use of *F. venenatum* as a food has led to the establishment of a history of safe use for this fungus.

Clearly, recombinant DNA technology has not only been used to improve enzyme production systems. Rather, by manipulating the metabolism of the fungus it has been possible to increase the yields of certain desirable metabolites (e. g., citric acid [12]), to eliminate unwanted metabolites (e. g., oxalic acid [13]), or to enable the production of metabolites that are "foreign" to the fungus (e. g., astaxanthin [14]). The manipulation of metabolism in this way is referred to as "metabolic engineering".

4.2 Additives for the Food Industry

Nowadays, the food industry uses additives of microbial origin for many different purposes, with enzymes being the best established examples of materials that are produced using genetically modified microorganisms (GMM) and used in food production.

In fact, enzymes have been used by the food industry for thousands of years. For example, chymosin isolated from calves has been used in cheese-making, amylases produced by barley have been used in brewing, and amylases and pectinases produced by grapes in wine-making. However, the deliberate use of microbial enzymes is a more recent development, and enzymes produced by GMM for the food industry were not introduced until the late 1980s. Depending on the country in which the enzymes are used, they are classified as either "food additives" or as "food processing aids".

Some examples of food industries that currently use enzymes on a regular basis include:

- Starch industry: this uses (by volume) the largest amounts of enzymes. Alpha amylases and amyloglycosidase are used for degrading starch into glucose, and part of the glucose syrup which is formed is further processed. Glucose isomerase is used to convert glucose into fructose for the production of high-fructose corn syrups.
- Baking industry: here, amylases and xylanases are used to improve the quality of bread by giving it more volume, a better crumb structure, and a longer shelf-life. More recently, lipases have been introduced to baking to provide *in situ* formation of emulsifiers.
- Diary industry: here, chymosin extracted from calves for cheese production has been supplemented with proteases of microbial origin. Chymosin produced by recombinant *A. niger* has also become a relatively important product. Other enzymes than rennet proteases are also used in the diary industry; for example, the microbial lactase which is used to hydrolyze lactose can benefit people suffering from lactose intolerance.
- Brewing industry: microbial amylases are used in the brewing industry to achieve a more robust and efficient mashing process. Enzymes have also been developed to remove the bad-tasting metabolite diacetyl from beer. However, as beer is seen

by many as a very traditional product, many countries have strict regulations on the type of additives which may be used in brewing.

- Wine industry: this is another example of a very conservative industry where the penetration of microbial enzyme use has been slow, and this is especially true for enzymes produced by recombinant microorganisms. Pectinases are used to increase juice yield during mashing, while β-glucanases are used to remove haze resulting from *Botrytis* growth on the grapes. A more curious use of enzymes in this industry is that of laccases to preserve cork stoppers, thereby preventing cork off-flavor in the wine.

4.3 Design of GMM for Production of Food Additives and Processing Aids

As enzymes are important examples of food additives and food processing aids produced using GMM, a description of the development of host strains and expression vectors for enzyme production is provided in the following section, together with a brief description of the tools used to produce metabolites.

As mentioned earlier, *A. oryzae* is one of the most important filamentous fungi for the recombinant production of enzymes. This fungus was selected as the host strain due to its history of safe use in products with GRAS status, to previous experience with the organism in production processes, and finally to the huge protein production potential of this microorganism.

The production of especially amylases and proteases by *A. oryzae* was a major drawback, however. Two primary requirements in enzyme production are product purity and product stability. When producing a particular enzyme, the presence of amylases is undesirable, not only because of the contaminating protein but also because amylase activity may cause unwanted side reactions. Likewise, any host strain proteases present may also cause contamination and negatively affect the stability of the enzyme product. Hence, these unwanted enzyme activities must – if possible – be totally removed, and the most important method to achieve this is gene disruption [15]. Gene disruption is also used to improve *A. niger*, *T. reesei* and *F. venenatum* expression systems.

4.3.1 Gene Disruption

The basic steps of gene disruption are:

1. The gene to be disrupted is cloned as a genomic clone together with 1–2 kilobases (kb) of upstream and downstream sequence (i.e., the 1000–2000 base pairs (bp) preceding the coding sequence of the gene and the 1000–2000 bp preceding the coding sequence).
2. A part (or all) of the coding sequence is replaced with a selectable marker to form the gene disruption plasmid.

3. The gene disruption plasmid is linearized (typically by digesting it with a restriction enzyme) and subsequently transformed into the fungus.
4. Transformants are recovered using the selection system of the disruption plasmid, after which the transformants are screened for the desired genotype.

The gene disruption event is outlined in Fig. 4.2.

The *pyrG* (orotidine-5'-phosphate decarboxylase) marker shown in the example is an auxotrophic marker that is widely used in *A. oryzae* and in other *Aspergillus* sp. for gene disruptions [16]. It is a biosynthetic gene in the pyrimidine pathway, and it is necessary that the strain to be transformed is *pyrG* negative. The advantage of this marker is that it is bi-directional; that is, selection for both presence and absence of the marker is possible. Selection for the presence of *pyrG* is carried out simply by transforming a *pyrG* negative strain and selecting on a minimal medium for pyrimidine prototrophy (only cells having an intact *pyrG* gene can grow on such plates). Selection for absence of the *pyrG* gene is carried out by plating on a minimal medium containing uridine and the compound 5-fluoro-orotic acid. The uridine addition will enable cells to grow without a functional *pyrG* gene, while the 5-flouro-orotic acid enables counter-selection for the

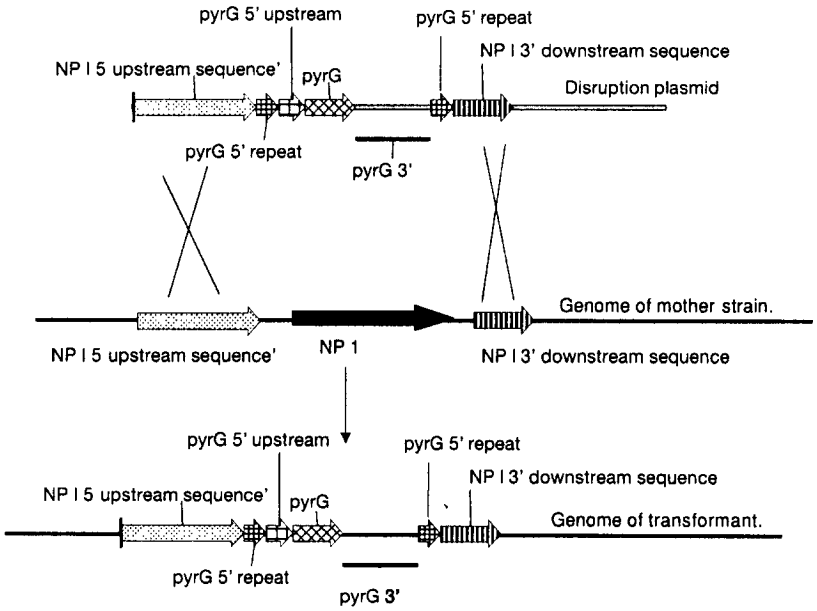

Figure 4.2 Gene disruption in filamentous fungi. The principle is illustrated by disruption of the gene NP I. A disruption plasmid was made by replacing the coding part of the NP I gene with the selectable marker *pyrG* (orotidine-5'-phosphate decarboxylase). The details of the components involved are described in the main text. In this example, a repeating sequence flanking the *pyrG* selection cassette has been included (*pyrG* 5' repeat). Recombination between the repeats results in the loss of the *pyrG* gene, and so these repeats greatly facilitate the selection of a *pyrG* negative strain with a predictable genotype for subsequent gene disruptions.

pyrG gene. This compound is converted into a toxic product by cells harboring a functional *pyrG* gene.

The bi-directional *pyrG* selection system is a very convenient tool for sequential disruption of several genes. A disruption plasmid is constructed for each of the genes that should be disrupted, and a *pyrG* negative mutant of the selected host strain is isolated using 5-fluoro-orotic acid. The *pyrG* mutant is then transformed with the first selection plasmid using selection for pyrimidine prototrophy. Transformants with the correct genotype, typically confirmed by Southern blot analysis [17], are then counter-selected for *pyrG*. The resulting strain is then ready for a new round of gene disruption using the next gene-disruption plasmid.

Other bi-directional markers are known, for example the *niaD* (nitrate reductase) system [18], but the *pyrG* system is the most applied system.

It is clear from Fig. 4.2 that the gene disruption event leads to major changes in the DNA sequence of the affected locus. These changes are irreversible, which means they will be present in all strains later in the pedigree of that particular host strain. They will thus also be present in transformants transformed with a particular product gene that is in the final GMM used for production. The gene disruption locus is thus an obvious target for analysis for the presence of DNA from the final GMM. In this way, recombinant host strain DNA may be detected, but if this host strain has been used for more products, it cannot reveal the specific product of the GMM.

By using gene disruptions, several amylase, glycoamylase, and protease genes have been disrupted in both *A. oryzae*, *A. niger* and *A. awamori*. A very similar technology has been used to disrupt primarily cellulase genes in *T. reesei*, metabolic pathways in *F. venenatum* [19], and in *A. niger* [13]. Rather than disrupt all of the individual genes, it is also possible to disrupt global activators that regulate the expression of entire classes of enzymes. For example, in *A. niger* a general protease regulator activates the expression of a range of extracellular proteases. Simply by disrupting this single gene, all of these proteases are silenced [20].

4.3.2
Expression Vectors

Development of the host strain is important in maintaining the efficiency of the expression system as well as the quality of the final product. Moreover, host strain improvement generates sequence tags in the final GMM suitable for analysis.

The expression vector is the other major factor in the final GMM, and is usually specific for the product to be expressed. It is therefore a natural target for analysis when analyzing for a specific product. In general, expression vectors comprise the following elements:

- The gene encoding the product to be generated.
- A promoter to drive expression of the product gene; this can be a promoter developed for the expression system (i. e., a generic promoter), or it can be specific to the product gene, typically the product gene's own promoter

- A translational terminator; as for the promoter, this can be a terminator generic for the expression system or it can be a specific terminator, typically the terminator of the product gene.
- A selection marker for selection in the expression host.
- Additional vector elements used to build the vector, for example an *Escherichia coli* selection marker and an *E. coli* origin of replication.

The expression vector is transformed into the fungal host cell, and then integrated into the chromosome of the host cell typically in more copies by tandem integration into one locus [21, 22]. An ideal integration of two copies is shown schematically in Fig. 4.3.

Typically, expression vectors are constructed in *E. coli*, and so elements necessary for this are often present in the final expression vector. The most widely used selection marker in *E. coli* is the β-lactamase gene which gives resistance to penicillins such as ampicillin. Public concern about the spread of antibiotic resistance markers has drawn attention to this part of the expression construct, even though antibiotic resistance markers from GMM used in contained production cannot be refound in nature, even when the biomass is spread to surrounding fields [23]. Hence, antibiotic markers have been replaced by other markers (e. g., auxotrophic markers) by most enzyme producers, and the *E. coli* portions of the expression vector are often completely removed before the construct is transformed into the host strain.

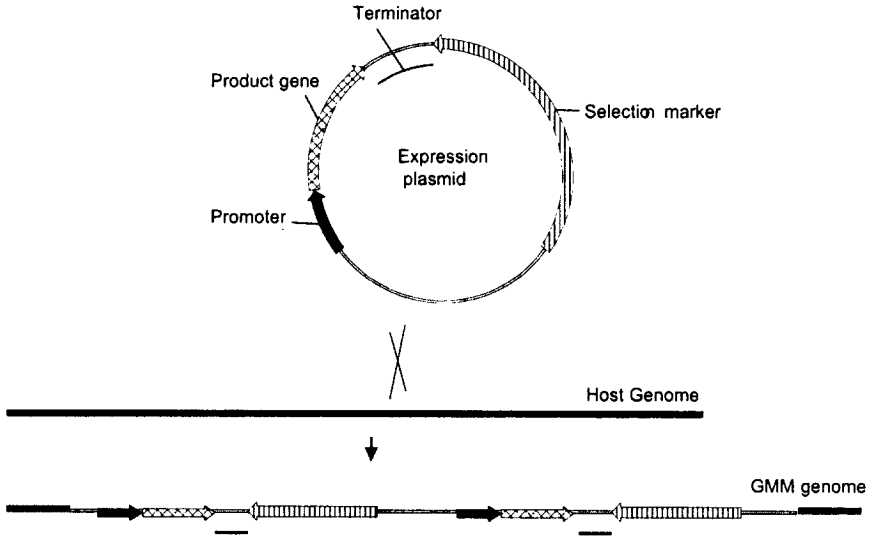

Figure 4.3 Integration of an expression plasmid into the genome. Tandem integration of an expression plasmid featuring a promoter, a product gene, a terminator and a selection marker into the genome of a filamentous fungus is shown schematically. The integration shown is head-to-tail in one locus, with no further recombination.

The choice of selection marker to select the expression vector in the fungus is very important, as different markers result in different ranges of copy numbers of integrated expression vector copies. The *pyrG* gene is sometimes used as a selection expression vector, and this is typically the case when the strategy is to insert the heterologous gene into a particular locus (perhaps the amyloglycosidase locus of *A. awamori*). The pyrG marker can also be used when the strategy is to remove the marker by recombination in a step following the transformation step, very much as described for gene disruption [24]. The drawback of these strategies is that typically only one copy of the expression vector is integrated into the genome, and this leads to low production yields.

The amdS (acetamidase) gene of *Aspergillus nidulans* [25] is often used as selection marker in *Aspergillus* sp., and even in other fungi. The selection principle is that the acetamide gene enables transformed cells to hydrolyze acetamide and, upon hydrolysis, to use the formed ammonium as sole source of nitrogen or the formed acetate as sole source of carbon. The advantage of this marker is that in both *A. oryzae* and *A. niger* high copy numbers are obtained, and thus high fermentation yields in production.

The choice of promoter is crucial for the yields, and this is one of the elements that has been most extensively studied and developed. For expression in *Aspergillus* sp., the amylase promoters were the natural choice as starting points for promoter development. These promoters are very powerful, and the ability to regulate them is very convenient in a production set-up as they are induced by starch, maltodextrins, and maltose. However, one of their drawbacks is that they are carbon catabolite-repressed, and this places a restraint on production. In order to avoid low productivity, the feed rate in fed-batch or in continuous fermentation must be carefully controlled. The *Aspergillus* amylase promoters are regulated very much in the same way, and they have been shown to be activated by the same activator [26].

The TAKA amylase is a very well-expressed amylase in *A. oryzae*, and hence the TAKA amylase promoter is one of the most widely used *Aspergillus* amylase promoters [8].

The neutral amylase promoter from *A. niger* is highly homologous to the TAKA promoter from *A. oryzae*, and is likewise broadly used [27]. These two promoters share sufficient sequence similarity to establish analytical methods that will be able to detect both.

The amyloglycosidase promoter from either *A. niger* or *A. awamori* is a promoter being in the same range of promoter strength. The sequence is quite distant from the sequence of the TAKA and the neutral amylase promoters, but it is regulated by the same activator. The amyloglycosidase promoter is also broadly used [9].

The choice of terminator is less critical than the choice of promoter. In *Aspergillus*, the amyloglycosidase terminator from *A. niger* is widely used [8].

The expression vectors for *T. reesei* are somewhat different than the *Aspergillus* expression vectors. The most frequently used promoter is the cellobiohydrolase I promoter, or variants of this [28]. This promoter is induced by cellulose-like sophorose, and also by more readily available carbon sources. This promoter is also carbon catabolite-repressed, but promoter variants where the creA sites mediating

this repression have been removed have been described [29]. Several different selection markers are used in *Trichorderma*; these are often dominant selection markers against drugs, for example the hygromycin or bleomycin resistance markers.

Various yeast systems have also been developed for the production of enzymes, including *Kluyveromyces lactis*, *Hansenula polymorpha* and *P. pastoris* [30]. One notable difference between the yeast systems and the filamentous fungal systems is that the yeasts have self- replicating plasmids; that is, the transforming DNA is not necessarily integrated into the genome of the host strain. Genetic stability using self-replicating plasmids is significantly lower than the stability of strains where the DNA has been chromosomally integrated, and so integrating expression vectors are also preferred in yeasts. Host strain development is carried out in very much the same way as for the filamentous fungi. In particular, proteases are removed by gene disruption. The methylotrophic yeasts *H. polymorpha* and *P. pastoris* produce large amounts of alcohol utilization genes when induced by methanol. All or some of these genes have been disrupted in some host strains, or the expression plasmid is integrated into one of these genes when making the final GMM, thereby disrupting the gene.

The promoters used to drive expression in the methylotrophic yeasts are typically promoters from the alcohol utilization genes such as alcohol oxidase 1 or formate dehydrogenase promoter in *P. pastoris*, or the methanol oxidase 1 or formate dehydrogenase promoter in *H. polymorpha*. In *K. lactis* the β-galactosidase promoter (*LAC4*) has been identified as a strong promoter. For all of the yeast systems several different selection markers including both dominant and auxotrophic markers have been developed. The *URA3* marker, the yeast homologue of *pyrG* is very often used.

The genetic manipulations for metabolites are very much of the same type as those described for enzyme production. The main tools in metabolic engineering are gene disruption, gene replacement, overexpression of genes (e. g., by increasing the copy number), or the introduction of new genes.

Gene replacement is a variant of gene disruption. The result of gene replacement is that a target gene is replaced with another gene, typically in a two-step strategy very similar to the gene disruption strategy previously described.

4.4
Industrial Enzyme Production Processes

Almost all industrial enzyme products are formulations of enzymes that are secreted from the fungus during fermentation. Following fermentation, the fungal biomass is removed and the enzyme recovered from the broth. As the GMM DNA is present in the biomass, it is removed together with the biomass. Hence, only GMM DNA that is released into the broth as a result of cell lysis, and which is neither removed nor degraded during the subsequent recovery and formulation processes, are present in the final product. This means that the utility of analysis on the final product for GMM based on the recombinant DNA will

be totally dependent on the production process. A brief introduction to industrial fermentation processes is provided in the following section.

Traditionally, enzyme production using filamentous fungi has been based on both surface fermentations and on submerged fermentation processes. Today, enzymes are produced almost exclusively by the latter approach.

The production of industrial enzymes by fermentation processes using recombinant fungi starts with the inoculation of a vial of the organism into a small flask containing an agar medium. The flask is then placed in an incubator that provides the optimal temperature for culture sporulation. The spores are then transferred to a seed fermenter (this is a small fermenter in which the biomass for the main fermentation is generated). Seed fermentation allows the cells to adapt to the environment and nutrients that they will encounter later on.

Following seed fermentation, the cells are transferred to the main fermenter, where temperature, pH and dissolved oxygen are carefully controlled to optimize enzyme production. The fermentation process can either be run as a batch process, a fed-batch process, or a continuous fermentation. In the batch process, all media components are added from the start of the fermentation, while in the fed-batch system the fungus is fed with additional medium during the fermentation. In a continuous fermentation, a steady state is reached by supplying fresh medium and harvest from the tank simultaneously. The degree of cell lysis during fermentation is mainly dependent on the organism that is fermented, the duration of the fermentation process, and the design of the fermenter (i. e., the shear stress that the biomass will encounter). A typical fermentation process is outlined in Fig. 4.4. When the main fermentation is complete, the mixture of cells, nutrients and enzymes – referred to as the "broth" – is ready for filtration, recovery and purification – this is collectively referred to as "downstream processing".

The next stage is to separate the broth containing the enzyme from the biomass. This is achieved by various chemical treatments of the fermentation broth to ensure efficient separation, followed by removal of the biomass using either centrifugation or filtration.

Following separation, the enzyme is concentrated by means of semi-permeable membranes or evaporation. In the case of products with high purity demands, the downstream process often requires special steps to remove unwanted impurities. This is often done by selective precipitation or adsorption of the impurities, or by crystallization by which very pure enzyme products can be obtained. In rare cases, a costly column chromatography step may be applied. The recovery process is outlined in Fig. 4.5.

The final step in the process is formulation of the enzyme product. The enzymes can be formulated either as liquid products or as granulates, depending on their application. The critical issues of the formulation are to secure stability of the enzyme product, release of the enzyme in the application, and to prevent enzyme dust formation that can cause allergy.

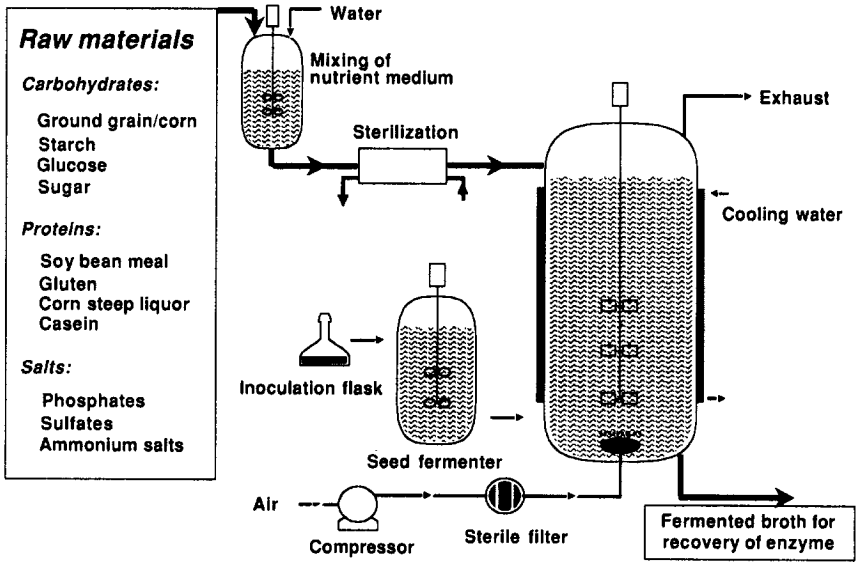

Figure 4.4 Schematic outline of a typical fermentation process flow. Spores from an inoculation flask are used to inoculate a seed fermenter, and this is in turn used to inoculate the main fermenter. The fermentation broth is aerated by stirring with large propellers. Additional medium components can be prepared and added during fermentation.

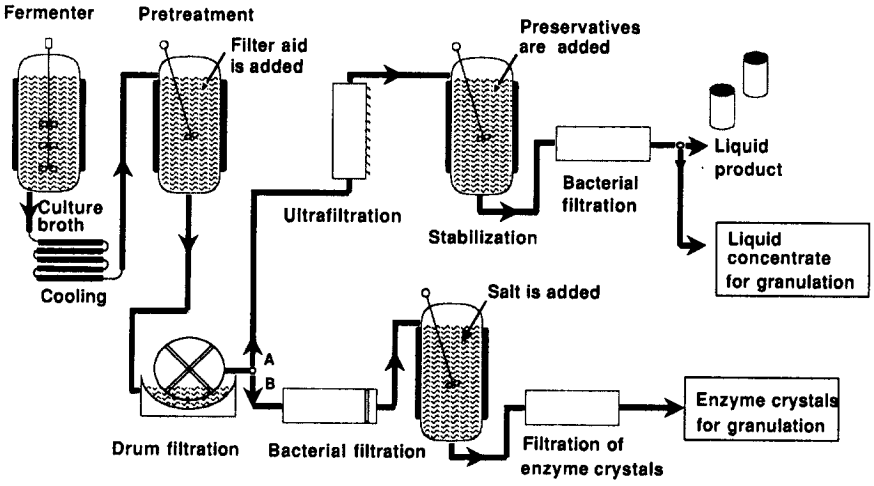

Figure 4.5 Outline of a recovery process. Typical process flows are demonstrated. The biomass is separated from the broth by filtration. The broth is then concentrated and sterile filtered, or alternatively the enzyme is recovered by crystallization.

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DTU

Analytical Report

Metabolite potential of *Trichoderma reesei* and *T. harzianum* strains

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CMB

1. Aim

To determine the ability of Novozymes strains *Trichoderma harzianum* (Ma-6) and *T. reesei* (Tv-10-6) to produce secondary metabolites and especially mycotoxins.

2. Literature

The genus *Trichoderma* is not one of the genera, which very often is associated with mycotoxin production. Since a *T. viride* strain was used for production of trichodermol and its acetyl ester trichodermin, the first trichothecenes to be fully structure elucidated (8,9), the genus has had reputation of being able to produce trichothecenes. However, only few publications on insufficient documented trichothecene production from *Trichoderma* exist, and presumably the only valid report is by Corley et al. (6) who found harzianum A from ATCC 90237 (=IBT 9471). We have confirmed the presence of this compound indirectly by GC-MS/MS of cultures of this strain, whereas 150 other *Trichoderma* cultures did not show any indication of trichothecene production (15). Reports of other trichothecenes as diacetoxyscirpenol (DAS) and T-2 toxin can be found in the literature, but we consider these results as false positives, as they never had been confirmed by third parties. Moreover DAS and T-2 toxin and other *Fusarium* trichothecenes are biosynthesised via iso-trichodermol (7) and not trichodermol as observed in *Trichoderma*.

Production of gliotoxin by *T. virens* (≡ *Gliocladium virens*) is well documented and has been verified in our laboratory. Several species of *Trichoderma* are able to produce cytotoxic peptaibols, which are polypeptides containing α-amino isobutyric acid. The extensive literature on these compounds has been summarized by (17).

The systematics of *Trichoderma* has developed rapidly within the recent 5-10 years as studies of DNA sequences in combination with classical techniques has resulted in splitting old species based on a broad species concept into a number of new species – see (5) for a recent summary.

3. Materials and methods

Strains were recieved from Novozymes in January 2004 and kept as frozen cultures until they were revived in June 2004 on oatmeal agar (OAT) where they appeared as morphologically fresh *Trichoderma* cultures. No further identifications were performed.

Together with IBT 9471 they were cultured on Yeast Extract sucrose agar (YES) agar, malt extract agar (MEA), OAT, potato dextrose agar (PDA), liquid potato dextrose medium, liquid YES, and liquid Raulin-Thom medium (appendix 1, other media see Samson et al. (16)).
Agar media were prepared in 9 cm Petri dishes and liquid media as 8 ml in 25 ml Bluecap bottles (loose lid) which were shaken at 200 RPM (13).

After 8 days at 25 C one colony was cut from the agar plate and extracted by shaking with 2 × 12 ml volume ethyl acetate over night. The liquid cultures were also extracted with 2 × 12 ml volume ethyl acetate by shaking over night. Next, the samples were centrifuged, and the ethyl acetate phases transferred to new vials, evaporated to dryness in vacuo and redissolved in 500 µl methanol.

Subsequently, the extracts were analysed with liquid chromatography–electro spray ionization - high-resolution mass spectrometry (LC-ESI-MS), first using negative ESI and secondly using positive ESI.

The LC was equipped with a Diode array Detector scanning from 200-700nm, and a Phenomenex (Torrance, CA) Luna II C_{18}, 3 μm particles, 50 × 2 mm column with a Phenomenex 2 mm C_{18} SecurityGuard pre-column.

Separation prior to ESI^+-TOF (Micromass LCT) was performed with water (MilliQ) containing 10 mM ammonium formiate and 20 mM formic acid (both analytical grade) and acetonitrile (AcN, gradient grade) containing 20 mM formic acid. A flow of 0.3 ml/min was used, starting with 5% AcN raising to 100% in 25 min, kept at 100% for 3 min, before returning to 5% AcN in 4 min. and then equilibrating for 7 min.
In ESI^+ two different skimmer potentials were used, 9 V and 39 V with a scan ranges of m/z 100-900 and 100-2000 respectively. A resolution of > 6000 (half peak height) was achieved. The lock spray (second spray) made on-line mass correction on leucine enkephaline every 3 sec (4,14).

In ESI^- the same gradient system was used with pure acetonitrile and water containing 100 ppm formic acid. Two different skimmer potentials were used, 10 V and 31 V both with a scan range of m/z 100-1000. A resolution of > 4500 (half peak height) was achieved. The lock spray (second spray) made on-line mass correction on 3,4-dihydroxybenzoic acid solution every 3 sec.

All major peaks from UV and MS traces were matched against reference standards in our metabolite collection (ca. 600) and the 341 metabolites reported from *Trichoderma* spp. in Antibase 2003 (Wiley).
Besides this, all ESI^- chromatograms were inspected for type B trichothecenes (nivalenol, fusarenon X and deoxynivalenol) and harzianum A, and all ESI^+ chromatograms for type A trichothecenes (trichodermol, trichodermin, T-2 toxin, HT-2 toxin, diacetoxyscirpenol, neosolaniol) and gliotoxins (A and Bis-dethio-bis(methylthio)-gliotoxin). For all these, except harzianum A, NMR validated reference standards were available. Harzianum A was tentatively identified in a culture of the known producer by correct accurate masses in ESI^- and ESI^+, appropriate fragmentations, and UV spectrum (Fig. 1).

4. Results

As shown in Table 1, no trichothecenes or gliotoxins or even indications of these were observed from any of the cultures under any of the used fermentation conditions.
However as shown in Fig.2, the *T. harzianum* isolate Ma-6 did produce a series of peptides which could be the trichotoxins (3,12), trichovirins, neoatroviridins, trichorzins (10,11). However specific identification of these will require rigorous tandem MS studies and perhaps also isolation and NMR spectroscopy.

Table 1. Detection of specific metabolites in the extracted ion chromatograms

		m/z (Da)	263	243	399	293	251	484	442	384	400	357	341	399	1650 to1750	800 to 2000
		Ion	Frag**	Frag**	$[M-H]^-$	$[M+H]^+$	$[M+H]^+$	$[M+NH4]^+$	$[M+NH4]^+$	$[M+NH4]^+$	$[M+NH4]^+$	$[M+FA]^-$	M+FA-	M+FA-	$[M+H]^+$, $[M+Na]^+$	$[M+H]^+$, $[M+Na]^+$
		RT (min)	5.70	5.90	10.3	9.0	5.4	9.32	7.33	6.28	2.33	0.71	1	1.66		
EK no.	NZ no.Species	Medium	Gliotoxin	Bis-glio*	harzianum A	Trichodermin	Trichodermol	T-2 toxin	HT-2 toxin	DAS***	NEO***	NIV***	DON***	F-X***	Trichotoxins	Other peptides
00222	Ma-6*T. harzianum*	YES-plate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	Perhaps, trace	Trace
00223	Ma-6*T. harzianum*	MEA-plate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	Perhaps, trace	Trace
00224	Ma-6*T. harzianum*	OAT-plate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	Perhaps, trace	Trace
00225	Ma-6*T. harzianum*	PDA-plate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
00226	Ma-6*T. harzianum*	Liquid PD	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
00227	Ma-6*T. harzianum*	Liquid YES	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
00228	Ma-6*T. harzianum*	Liquid RT	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	Perhaps	YES
00229	Tv-10-6*T. reesei*	YES-plate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	YES
00230	Tv-10-6*T. reesei*	MEA-plate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	
00231	Tv-10-6*T. reesei*	OAT-plate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	YES
00232	Tv-10-6*T. reesei*	PDA-plate	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	High quant
00233	Tv-10-6*T. reesei*	Liquid PD	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
00234	Tv-10-6*T. reesei*	Liquid YES	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	YES
00235	Tv-10-6*T. reesei*	Liquid RT	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	High quant

*Bisdethio-bis(methylthio)-gliotoxin
** Fragment from ESI positive MS
*** Standard abbreviations for trichothecenes.

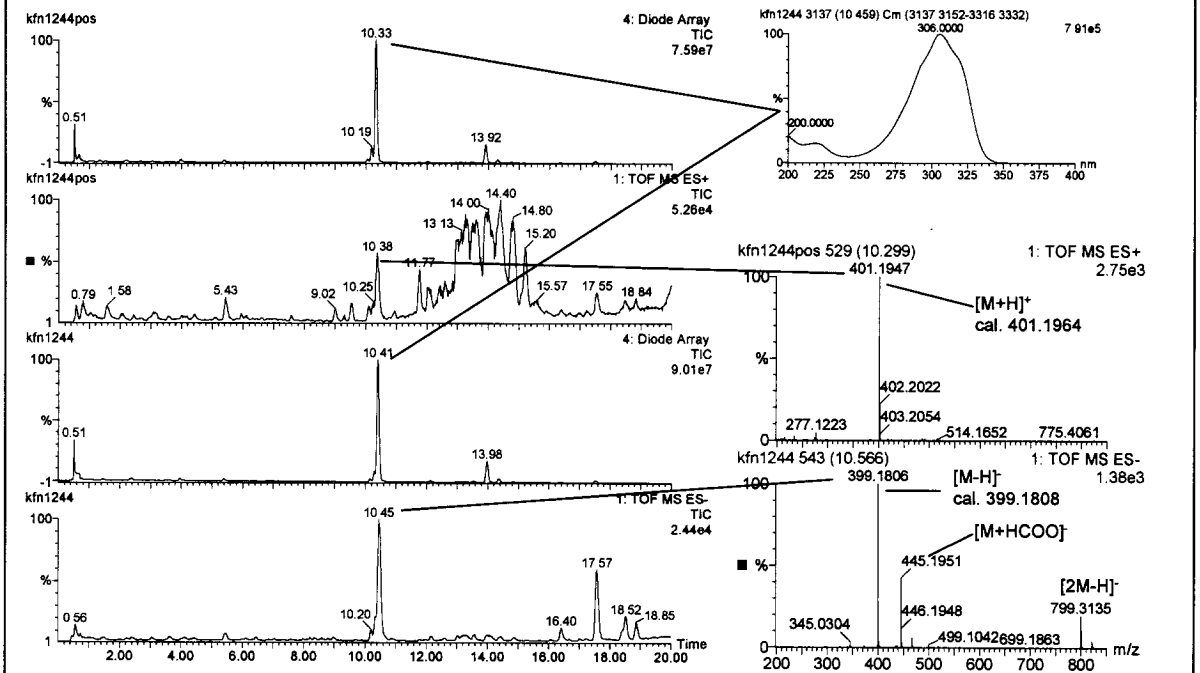


Fig. 1. Dereplication of harzianum A from T. harzianum *IBT 9471 also used by Corely et al for isolation of this compound.*

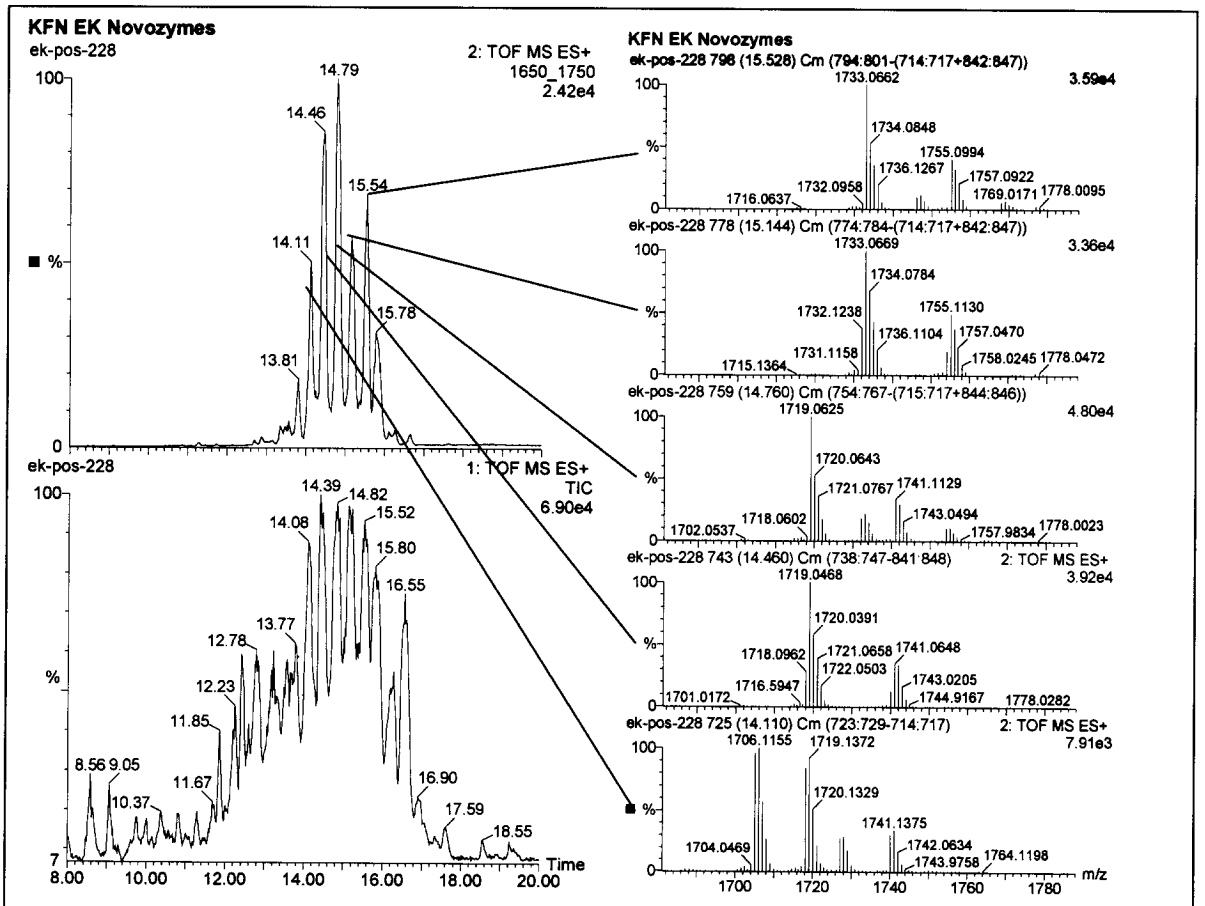


Fig. 2. Chromatograms of T. harzianum Ma-6 (Raulin Thom medium), with the upper showing m/z1650 to 1750 which is the region where the trichotoxins have their molecular masses, below is the total ion chromatogram. Mass spectra of the individual peaks are shown to the right.

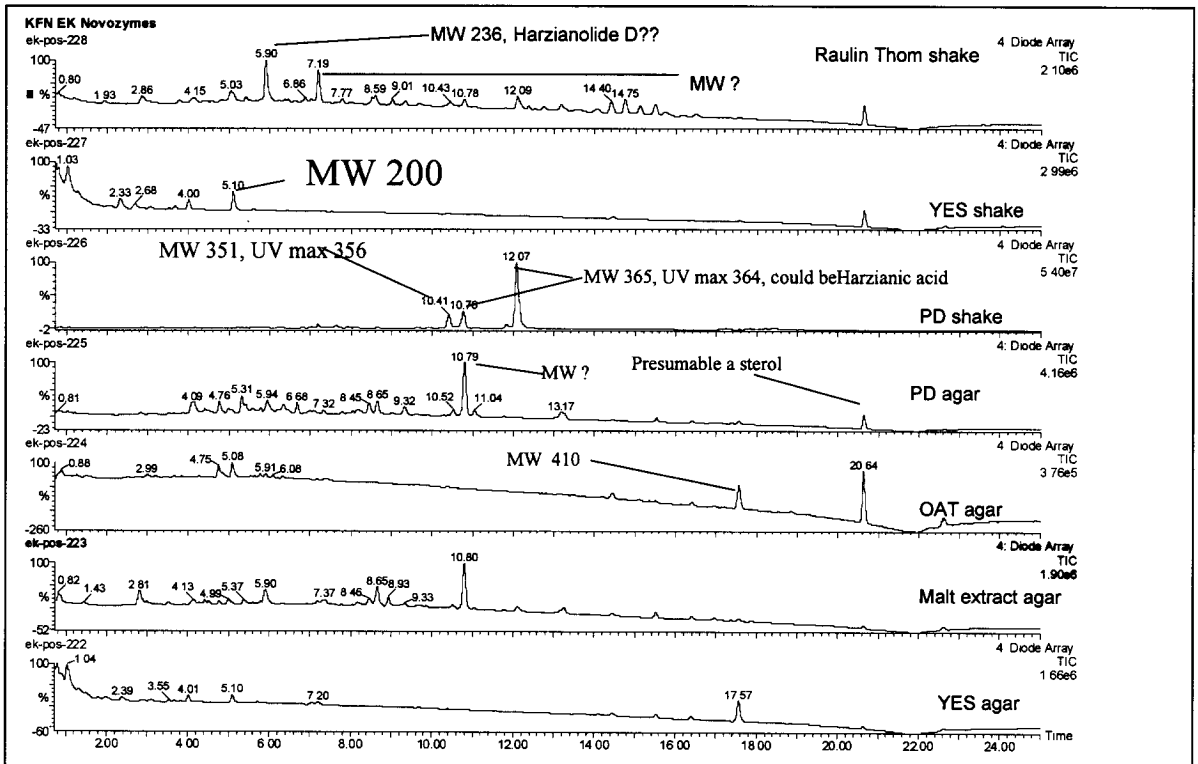


Fig. 3. UV Chromatograms of extracts of T. harzianum Ma-6 grown on various media, with tentative ID on metabolites not available as reference standards shown.

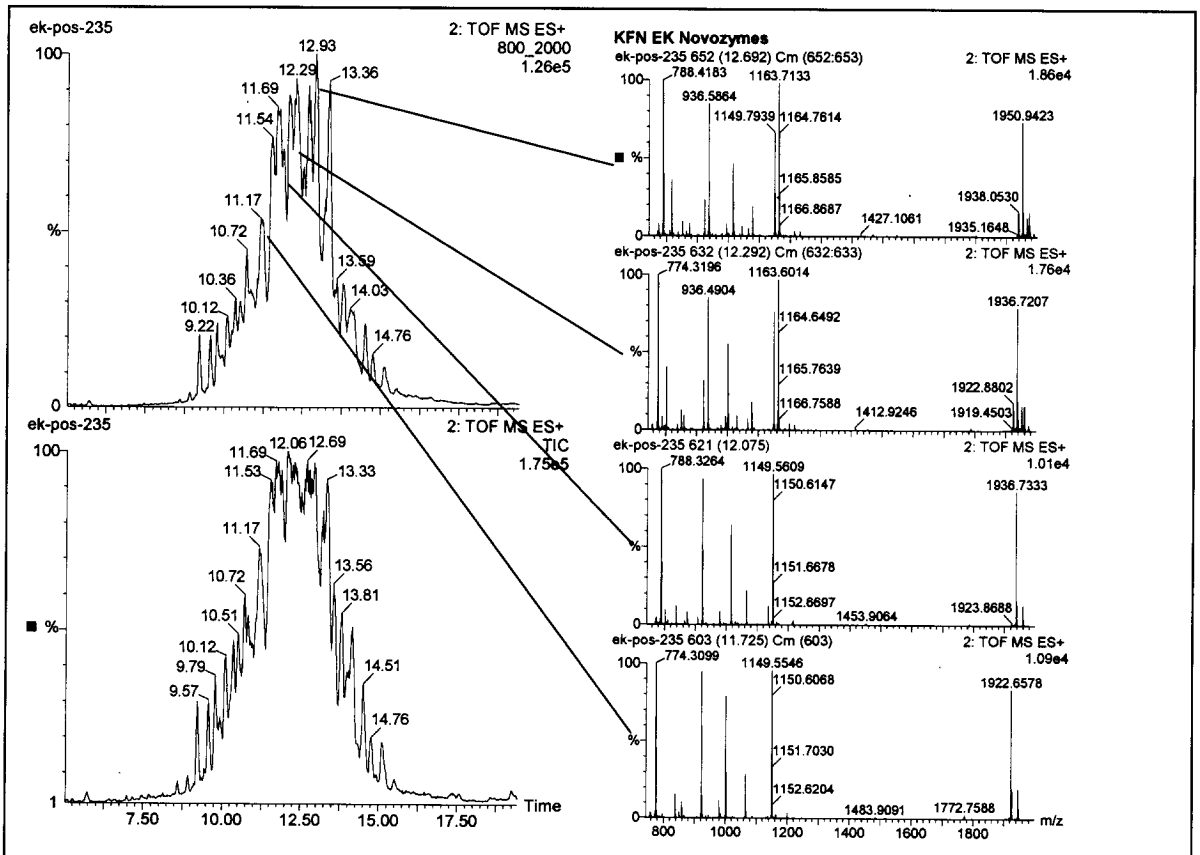


Fig. 4. UV Chromatograms of extracts of T. reesei Tv-10-6 (Raulin Thom medium), with the upper showing m/z 800 to 2000 which is the region where most peptides elutes, below is the total ion chromatogram. Mass spectra of the individual peaks are shown to the right.

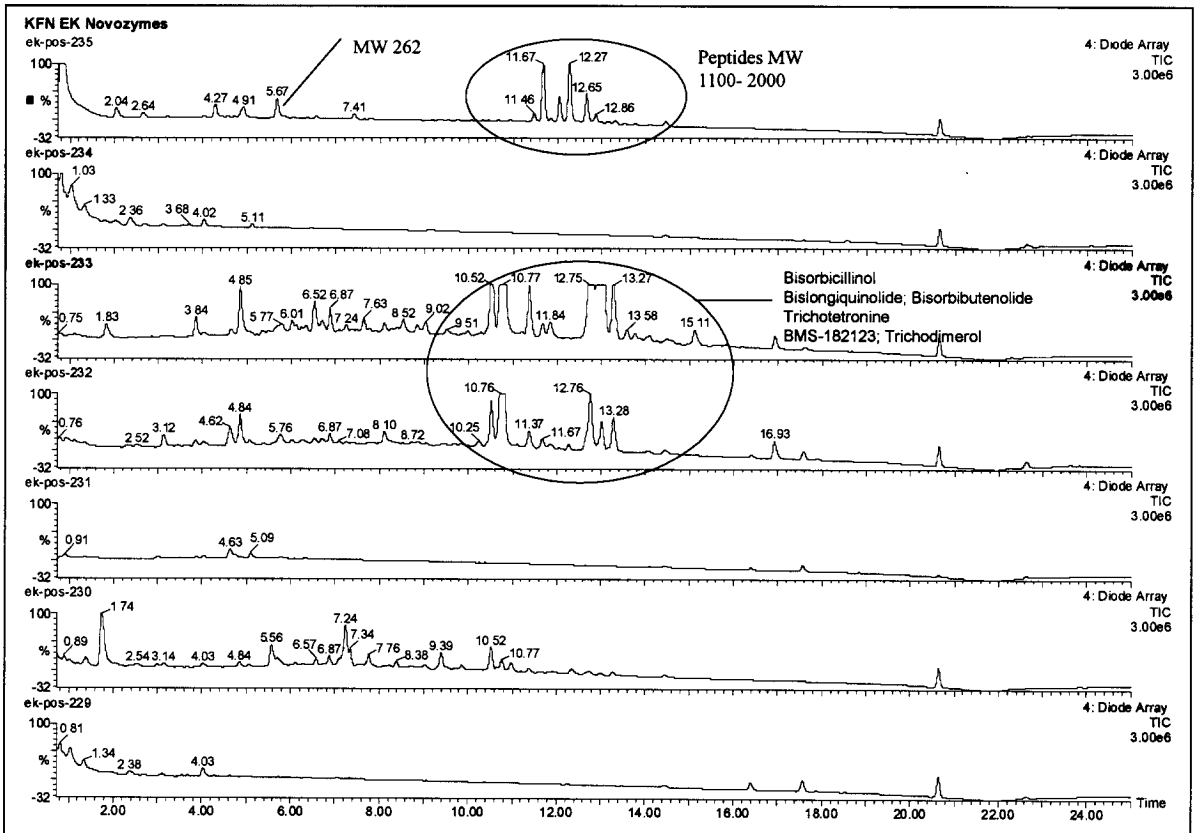


Fig. 5. UV Chromatograms of extracts of T. reesei Tv-10-6 grown on various media, with tentative ID on metabolites not available as reference standards shown.

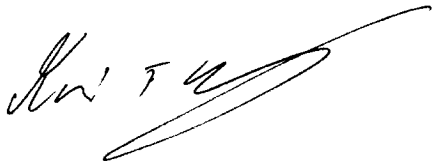
Besides the possible trichotoxins, the *T. harzianum* only produced very low quantities of metabolites and none of these could be matched in our reference standard database, and only two of the three matched possible metabolites in Antibase (Fig 3.), as MW 236, Harzianolide D and Harzianic acid.

From *T. reesei* T10-6 a number of peptides were detected (see fig. 4) with molecular masses from ca. 1150 Da to almost 2000 Da (upper scan range so they could be bigger); however, for the ions close to 2000 Da. it does not make sense to match these data in Antibase as it contains 198 metabolites > 800 Da from *Trichoderma* (>50% of the described metabolites from this genus).

Besides the peptides, the *T. reesei* strain produced a series of sorbicillin derivatives with very distinct UV resembling bisorbicillinol, bislongiquinolide; bisorbibutenolide, BMS-182123 (=Trichodimerol) known from *T. longibrachiatum* (2) and an undentified *Trichoderma strain* (1)

5. Conclusion

No trichothecenes or gliotoxins or even indications of these were observed from any of the two cultures under any of the used fermentation conditions, although these were chosen to induce as many secondary metabolites as possible.
The *T. harzianum* isolate Ma-6 produced a series of peptides, which could be the trichotoxins or related peptides, whereas the peptides detected in cultures of *T. reesei* Tv-10-6 could not be identified.



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Appendix A

RT Raulin Thom	(farvekode:rød, gul, rød)
$(NH_4)\ H_2PO_4$ (Merck, 1126)	0,4 g
K_2CO_3 (Merck, 4928)	0,4 g
$ZnSO_4,7H_2O$ (Merck, 8883)	0,06 g
$FeSO_4,7H_2O$ (Merck, 3965)	0,06 g
$(NH_4)_2So_4$ (Merck, 1217)	0,16 g
$MgCO_3$ (Riedel-de Haën, 13118)	0,25 g
$C_4H_6O_6$ (Vinsyre) (Merck, 804)	2,6 g
$C_4H_{12}N_2O_6$ (di-Ammonium tartrat) (Merck, 1222)	2,6 g
Glucose (D+) (BHD, 10117)	50,0 g
Agar (Bie & Berntsen,BBB10030,SO-BI-Gel,Agar-Agar)	20,0 g
Spor metal opløsning (SM)	1 ml
Vand, dobbelt, dest.	1000 ml